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V. THERMOSTABLE CELLULASE ENZYMES FROM ACIDOTHERMUS CELLULOLYTICUS: STUDIES WITH GROWTH MEDIA ACTIVITIES

Melvin P. Tucker, Ali Mohagheghi and Michael E. Himmel

INTRODUCTION

One result of the uncertainties in the fossil-fuel market experienced in the last decade is a renewed interest in the production of liquid fuels via fermentation from woody biomass and low-value agricultural residues. Modern process designs for the depolymerization of cellulosics focus either on acid or enzyme based catalysts. Acid treatments proceed with rapid rates of conversion (Goldstein et al, 1983), but suffer either high catalyst consumption in concentrated acid processes or low sugar yields in high temperature dilute acid processes due to sugar destruction. Enzymatic hydrolysis is more favorable, since this catalyst is noncorrosive, non-environmentally hazardous, reusable, and offers an efficiency of better than 90% (Cuskey et al, 1982, and Deshpande et al, 1984).

An historical drawback to the use of cellulase preparations from mesophilic fungi or bacteria was the limited solution lifetimes exhibited by these enzymes at elevated temperatures (Ryu et al, 1980). Cellulase enzymes from thermophilic microorganisms, however, may be applied to process streams at elevated temperatures (e.g., hot pulps from dilute-acid cooking of biomass) where characteristics such as higher kinetic rates, reduced cooling costs and reduced contamination are economically attractive. Also, experimental evidence (Daniel et al, 1982) has confirmed the notion that thermally stable enzymes are also more resistant to other types of denaturation, including protease digestion, shear and foam sensitivity, and inactivation by organic and ionic agents.

We have recently reported (Mohagheghi et al, 1986) the isolation of Acidothermus cellulolyticus gen. nov., sp. nov., a moderately thermophilic, aerobic, cellulolytic bacterium, from the acidic hot springs of northern Yellowstone National Park, Wyoming. In the present study we report that the endo-beta-1,4-glucanase (EC 3.2.1.4), beta-glucosidase (EC 3.2.1.21) and saccharifying cellulase activities produced by this bacterium possess the highest temperature optima reported to date.

Acidothermus cellulolyticus ATCC 43068 was found to grow optimally at pH 5.5 and 55°C utilizing a wide variety of simple sugars and/or cellulose as carbon source in minimal media (Mohagheghi et al, 1986). These conditions for cell growth were found, not surprisingly, to closely resemble those discovered at the hot spring site itself. When grown in the laboratory on minimal media with trace addition of yeast extract in the presence of cellobiose or a microcrystalline cellulose such as Sigmacell 50 (Sigma Chemical Co.), these cultures actively secrete a cellulase enzyme complex producing cellobiose as the final product.

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EXPERIMENTAL

For studies of thermal tolerance, the A. cellulolyticus microorganism was grown on 0.3 wt% cellobiose in 120 L culture using a New Brunswick 150L fermenter. The growth media from late-log culture was harvested by continuous centrifugation using a CEPA Z41 separator and the clarified supernatant was concentrated 140-fold and exchanged with 20 mM acetate buffer, pH 5, using an Amicon DC-30 hollow fiber ultraconcentrator fitted with polysulfone membranes (10,000 M_r cutoff). This fraction, the secreted enzyme fraction, was then used directly for total saccharification (filter paper) and CMC degrading activity studies. The cell pellet (approx. 770 grams) was halved and added to 150 mL of 50 mM acetate buffer, pH 5. This slurry was subjected to the shear induced by decompression using a French press (Parr Inc.) operating at 14,000 psi, after which the cell debris was pelleted by centrifugation. The supernatant from this step was fractionated by ammonium sulfate precipitation where the beta-glucosidase activity was found in the 40 wt% ammonium sulfate suspension. After centrifugation, this pellet was then leached with 100 mL of 50 mM acetate buffer and the resulting liquor used for the beta-glucosidase

The thermotolerance of enzymes is traditionally determined in two ways. One approach is to perform the assay (described in detail in legend to Figure 1) with the same substrate-incubation time period and enzyme loading (substrate is always in excess) and vary the temperature of exposure during this incubation. The second approach is to maintain the enzyme solution at various temperatures for various lengths of time (referred to as pre-incubation) and remove aliquots of enzyme periodically for addition to substrate and subsequent analysis at a common temperature (here 65°C) and incubation time. These methods are, of course, related ways of examining the contributions of both the rate of enzymatic denaturation (as activity loss) and the rate of catalysis enhancement as a function of temperature.

RESULTS AND DISCUSSION

In Figure 1 the effectiveness of the first approach in illustrating the apparent activity optima of the three cellulase activities found in supernatants and the cell-cytosolic fraction of A. cellulolyticus can be observed. This figure shows the dramatic loss of beta-glucosidase activity (pNPG as substrate) after reaching 75°C, whereas the filter paper (FP) and CMC degrading activities gradually decrease in activity after reaching the maximum condition at 75 and 83°C, respectively. Also, the very broad activity/temperature curve found for the CMC degrading activity, where a 50% or higher activity level was observed between 37 and 95°C, is unusual.

The effects of preincubating the concentrated growth supernatant at various temperatures on cellulase activity are shown in Figure 2. In general, the CMC degrading activity shown in this figure appears to decrease in a less abrupt manner than does the FP degrading activity. This behavior may signal the requirement of two or more enzymes for FP degradation and the thermal sensitivity of one of these components not involved in CMC hydrolysis.

The importance of these findings is well illustrated by the data shown in Table 1. The literature review represented by this table revealed that the

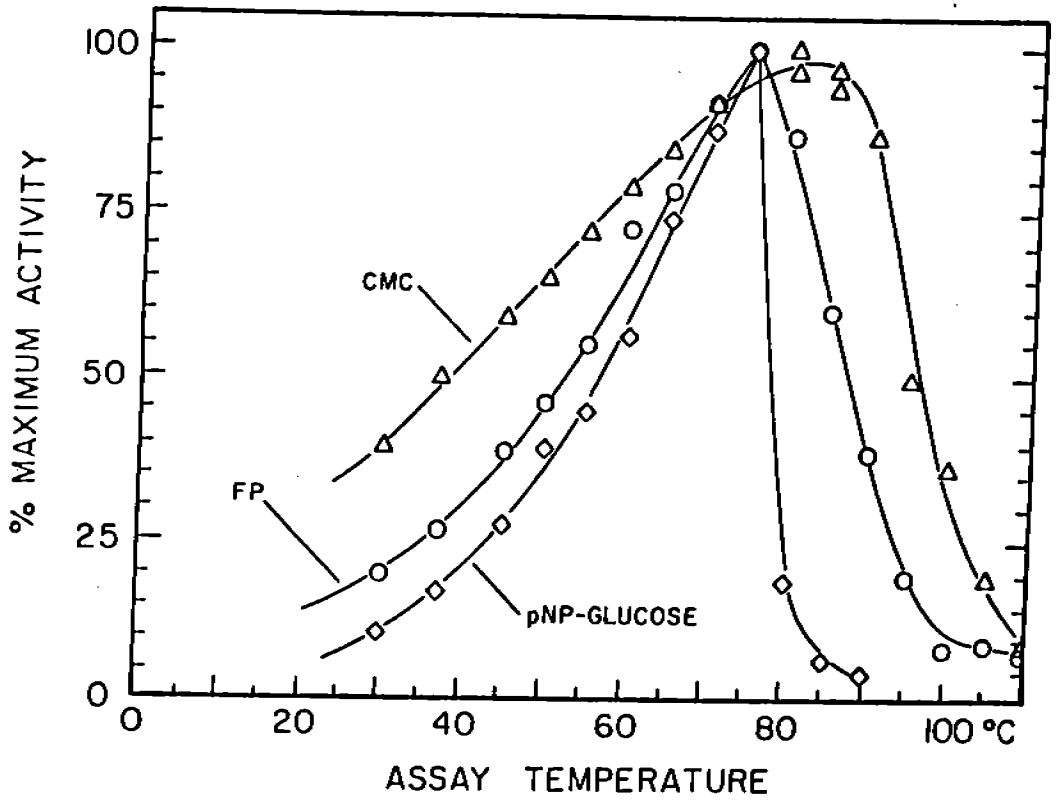

Figure 1. Comparison of the percent maximum filter paper (FP) and carboxymethylcellulose (CMC) degrading activities found for *A. cellulolyticus* growth supernatant at different assay temperatures. Beta- glucosidase activity was determined by the hydrolysis of p-nitrophenyl-beta-D-glucoside (pNPG), and was found only in the cell-cytosol fractions (See text). Assays for CMC and FP degrading enzymes followed the methods of Sternberg, Vijaykumar, and Reese (1977) and Mandels, Andreotti and Roche (1976), as modified in an IUPAC report (Ghose et al, 1984). Here, one CMC degrading unit equals that amount of enzyme liberating 1 micromol glucose from CMC (7LF, Hercules Inc.) per minute and one FP unit of activity equals that amount of enzyme complex yielding 1 micromol glucose per minute from 50-mg strips of Whatman No. 1 filter paper. Under the recommended conditions of the CMC and FP degrading assays, enzyme/substrate ratios must be adjusted so that 0.5 mg and 2.0 mg glucose is released per 30 minute and one hour incubations, respectively. The beta-glucosidase was determined by the method of Wood (1971) as aryl-beta-glucosidase by the hydrolysis of pNPG (Sigma Chemical Co.).

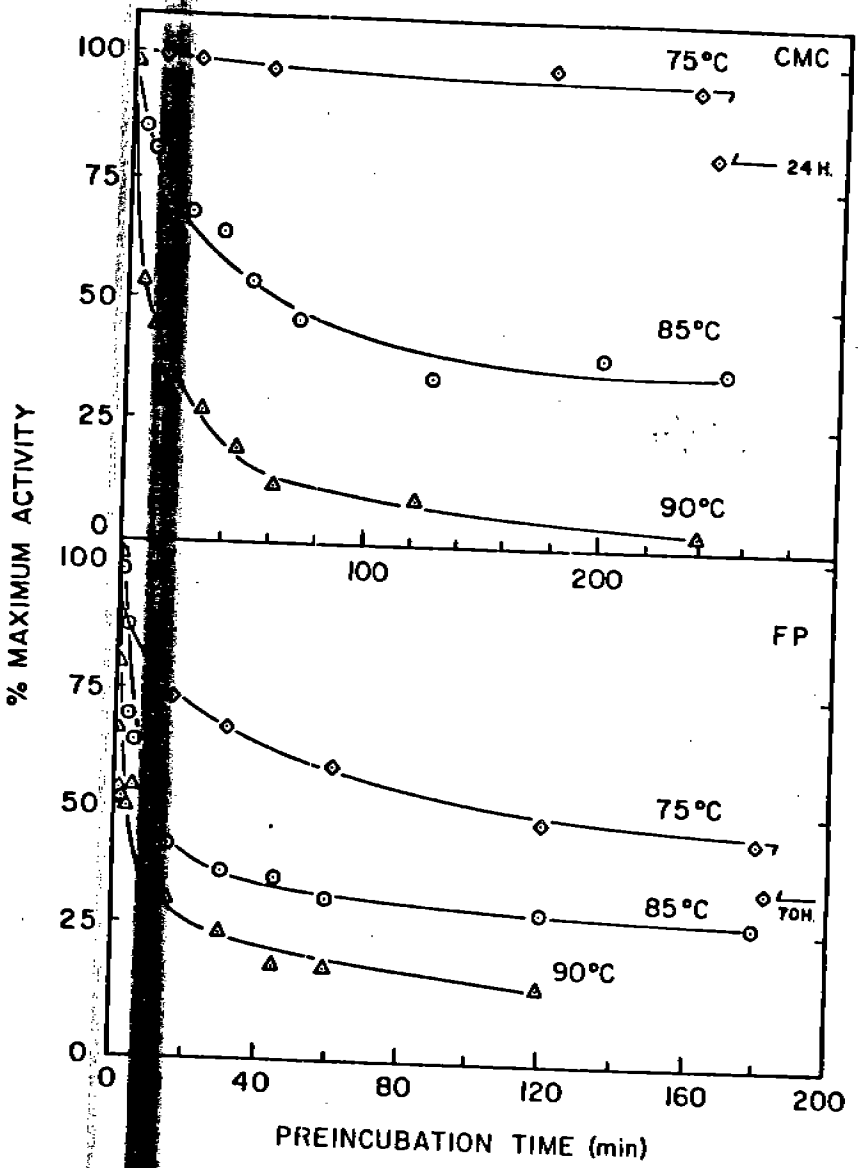

Figure 2. ...e course studies of the decline in FP and CMC degrading ac...ities for *A. cellulolyticus* growth supernatant samples as a fu...tion of preincubation temperature.

TABLE 1

TEMPERATURE (°C) OPTIMA REPORTED FOR CELLULASE COMPLEX ACTIVITIES

MICROORGANISMS	SUBSTRATES			
	FILTER PAPER	CMC	pPNG	REF.
Trichoderma reesei QM6a	45[a]	55[c]	NR	Mandels,1978
Penicillium sp. PD 20	55[a]	55[c]	NR	Durand,1984
Trichoderma reesei TD1 beta 6	55[a]	60[c]	NR	Durand,1984
Chaetomium thermophile var. dissitum	59[i]	71[h]	NR	Eriksen,1977
Sporotrichum cellulophilum #	60[a]	60[c]	60[c]	Durand,1984
Thielavia terrestris NRRL 8126 #	60	60	60	Durand,1984
Thielavia terrestris ATCC 26917	60[b]	70[f]	70[g]	Margaritis,1983
Clostridium thermocellum LQ8	64[n]	65[c]	NR	Ng,1977
Sporotrichum thermophile SN-16	65[b]	60[c]	65[d]	Awa,1984
Thermomonospora sp. YX strain	65[k]	70[f]	55[e]	Hagerdal,1980
Thermoascus aurantiacus	65[l]	70[c]	NR	Tong,1982
Talaromyces sp. 207	65[b]	70[c]	70[e]	Liu,1984
Myceliopthora thermophile D14	65[j]	70[h]	60[d]	Sen,1982
anaerobic bacterium TP8	NR	70[c]	NR	Reynolds,1986
Thermoascus sp. 239	70[b]	75[c]	75[e]	Liu,1984
Clostridium thermocellum ATCC 27405	70[m]	70	NR	Johnson,1982
Acidothermus cellulolyticus 11B	75[b]	83[c]	75[d]	this study

CMC, NR, and # indicate carboxymethylcellulose, not reported, and patented strains (Skinner et al, 1978), respectively. Although the above data have been normalized to like units of activity, the specific conditions of assay do vary widely (i.e., a = disk assay (Montenecourt et al, 1978); b, j, and l = filter paper hydrolysis (Ghose, 1984) with 60, 120 minutes, and 24 hours incubation, respectively; f, c, and h = CMC hydrolysis (Ghose et al, 1984) with 10, 30, and 60 minutes incubation, respectively; g, e, and d = pNPG assays with 10, 30, and 60 minute incubations, respectively; i = cotton hydrolysis with 60 minute incubation, and k, n and m = Avicel hydrolysis with 20, 120 minute incubations and by Avicel turbidity assay, respectively.

search for thermostable cellulase enzymes has a long, vigorous history and has been conducted with soil and compost samples from Taiwan, China and France and with hot spring sediment samples in New Zealand, Iceland and America (Yellowstone Park). Also notable from Table 1 was the wide distribution of temperature optima reported for filter paper degrading enzymes (i.e., a 30°C span). Another trend important to others looking for new thermotolerant cellulase producers was the polarization of bacterial cellulases at the higher temperature optima, even though many more thermotolerant fungi are known.

Perhaps the most striking feature of cellulases derived from the Yellowstone hot spring microorganism is shown in Table 2. This table is designed to compare the preincubation time required to give a 50% activity loss at temperatures between 60 and 100°C for thermostable cellulases reported in the literature. The lack of universal guidelines for conducting these studies leads to the somewhat incomplete data shown in Table 2, however, the data retrievable from the literature provides an interesting picture. The wild-type Trichoderma reesei (designated QM9414 from the military uniform of quarter master no. 9414 - U.S. Army Natick Labs) shows a one hour FP half-life (minutes) inactivation occurs at 70 and 75°C for the CMC and FP degrading activities, respectively. The "improved" fungal strains Trichoderma reesei TD_1 beta6 and Thielavia terrestris NRRL 8126 are no better (Durand et al, 1984) at temperatures above 60°C, yet have significant improvement in half-life at 60°C. Three bacteria secrete enzyme systems which show the most impressive stabilities as a function of preincubation at elevated temperatures. These are: Clostridium thermocellum JW20, a well studied (Hon-Nami et al, 1985) thermophilic, anaerobic bacterium; Microbispora bispora, a newly discovered thermophilic actinomycete (Waldron et al, 1986); and A. cellulolyticus (reported in this study). All three bacterial cellulases posses activity stability so high at 60°C that 50% loss in starting activity cannot be easily achieved. However, the CMC degrading enzymes from C. thermocellum are inactivated at 80°C in 6 minutes. Also, this temperature is sufficient to cause a 50% reduction in CMC activity of the enzymes from M. bispora after 18 minutes. The A. cellulolyticus CMC degrading enzymes are still very stable at 75°C and show half-lives of 60 and 12 minutes at 85 and 90°C, respectively. M. bispora is also noteworthy, in that these CMC degrading enzymes showed a half-life of 12 minutes at 100°C, however at this temperature they were inactivated soon after this period of time. The CMC degrading enzyme complex of A.cellulolyticus appears to be inactivated after four hours at 90°C, however maintenance of activity at the 45% level at 85°C is very strong (See Figure 2). Also, the FP degrading enzyme complex produced by A. cellulollyticus remains active (30% level) for 3 days and longer at 75-85°C.

An important observation taken from Table 2 is that only A. cellulolyticus produces FP degrading enzymes which have been reported to have high thermostability, which characteristic is the key to industrial interest where total saccharification of cellulose is the target.

Examination of the literature values reported for filter paper activity versus cell-growth temperature optima shows the following differences in thermal optima; T. reesei (Coughlan et al, 1985) +12°C, Thermomonospora sp. (Coughlan et al, 1985) +10°C, T. aurantiacus (Tong et al, 1982) +20°C, M. thermophile (Sen et al, 1982) +15°C, C. thermocellum (Duong et al, 1985) +8°C,

Table 2

Cellulase Activities From Crude Culture Broths

°C	*Trichoderma reesei* QM9414 (21)	*Trichoderma reesei* TD_1 beta6 (14)	*Trichoderma terrestris* NRRL 8216 (14)	*Clostridium thermocellum* JW20 (7)	*Microbispora bispora* (28)	*Acidothermus cellulolyticus*
	CMC/FP	CMC/FP	CMC/FP	CMC/FP	CMC/FP	CMC/FP
60	NR/1	36/3	72/56	v. high	v. high	v. high
65	1/NR	NR/NR	NR/NR	NR/NR	NR/NR	unknown
70	INAC./NR	INAC.	INAC.	5/NR	NR/NR	unknown
75	/INAC.			0.4/NR	NR/NR	v. high/3
80				0.1, INAC./NR	0.3/NR	unknown
85					NR/NR	1/0.2
90					NR/NR	0.2/0.1
95					NR/NR	unknown
100					0.2, INAC./NR	unknown

Preincubation Temperature

$T_{50\%}$ (hours)*

*Time required at preincubation temperature to give 50% activity loss on the cellulase substrate, CMC (carboxymethylcellulose) and FP (filter paper). V. high indicates T_{50} not readily measurable due to high stabilities. NR and INAC. indicate data not reported and temperature at which inactivation occurs in less than 60 minutes.

and A. cellulolyticus +20°C. We conclude from these data that extreme cell thermophily is not a requirement for the evolution of enzymes providing extreme thermostability. Although the reasons for macromolecular- versus cell-stability at extreme temperatures are still under formulation (Amelunxen et al, 1978 amd Margaritis et al, 1986), the discovery of new, thermotolerant enzymes increases the current data base and thus positively impacts basic and applied sciences alike.

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VI. PURIFICATION AND PARTIAL CHARACTERIZATION OF TWO ENDOGLUCANASES FROM ACIDOTHERMUS CELLULOLYTICUS

Melvin P. Tucker and Michael E. Himmel

INTRODUCTION

The goal of this study was to further understanding of bacterial cellulase systems through the isolation and characterization of the principal enzymes involved in the saccharification process. The new thermotolerant bacterium Acidothermus cellulolyticus was chosen for this study as it has potential for commercial application and secretes highly thermostable cellulase enzymes. This system was found to consist of two key components, a high molecular weight enzyme (EGH) and a low molecular weight enzyme (EGL).

RESULTS AND DISCUSSION (CONDENSED)

Preliminary substrate specificity analysis has shown that both enzymes exhibit activity on carboxymethylcellulose (CMC), filter paper, and phosphoric acid swollen cellulose. These characteristics, in the absence of further data, result in the classification of these enzymes as "endoglucanases".

The EGL was purified to homogeneity from fermentation supernatant by preparative size exclusion chromatography (Fractogel 55S) followed by preparative anion exchange chromatography using the Pharmacia FPLC system (Mono Q 10/10). EGL was found to migrate as a single band on SDS-PAGE and elutes as a single, symmetrical peak on analytical HPSEC (TSK 3000 SW). The non-denaturing HPSEC method provided a native enzyme molecular weight of 72,000 and the denaturing/reducing SDS gel showed it has two subunits of 32,000 daltons each. Isoelectric focusing experiments showed the isoelectric pH of EGL is 7.1. The data presented above allow interesting comparisons with fungal endoglucanases. The EGL from Acidothermus is of similar molecular weight to the T. reesei enzymes, as EGI and EGII from that source are 55,000 to 60,000 MW.

EGH, the high molecular weight endoglucanase from Acidothermus, was partially purified using methods similar to those described above for EGL. The isoelectric pH of EGH was also similar to, yet slightly more acidic than the EGL. Although the EGH sample elutes from analytical HPSEC as a single, symmetrical peak at a volume corresponding to 220,000 daltons, when examined of SDS-PAGE it migrates as two bands, one at 120,000 and one at 60,000. These data indicate that EGH exists as a tetramer of 220,000 under native conditions (i.e., fermentation broth). When exposed to denaturing and reducing conditions, however, it is only partially degraded to component subunits. The 120,000 and 60,000 MW components are clearly not contaminating proteins, as they are not observed in the size exclusion chromatography study. It is unusual, however, for multimeric enzymes to denature only partially as observed here, that is, to have tetramer, dimer